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Photosynthesis and Activation of Ribulose Bisphosphate Carboxylase in Wheat Seedlings[1]

REGULATION BY CO_2 AND O_2

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ABSTRACT

Photosynthetic carbon assimilation in plants is regulated by activity of the ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase. Although the carboxylase requires CO_2 to activate the enzyme, changes in CO_2 between 100 and 1,400 microliters per liter did not cause changes in activation of the leaf carboxylase in light. With these CO_2 levels and 21% O_2 or 1% or less O_2, the levels of ribulose bisphosphate were high and not limiting for CO_2 fixation. With high leaf ribulose bisphosphate, the $K_{act}(CO_2)$ of the carboxylase must be lower than in dark, where RuBP is quite low in leaves. When leaves were illuminated in the absence of CO_2 and O_2, activation of the carboxylase dropped to zero while RuBP levels approached the binding site concentration of the carboxylase, probably by forming the inactive enzyme-RuBP complex.

The mechanism for changing activation of the RuBP carboxylase in the light involves not only Mg^{2+} and pH changes in the chloroplast stroma, but also the effects of binding RuBP to the enzyme. In light when RuBP is greater than the binding site concentration of the carboxylase, Mg^{2+} and pH most likely determine the ratio of inactive enzyme-RuBP to active enzyme-CO_2-Mg^{2+}-RuBP forms. Higher irradiances favor more optimal Mg^{2+} and pH, with greater activation of the carboxylase and increased photosynthesis.

Previous studies by this laboratory and others (11, 13) indicate that the activity of the RuBP[3] carboxylase as expressed in the leaf is a major factor regulating photosynthesis. We have reported that irradiances greater than 225 $\mu E(m^2 \cdot s)^{-1}$ during steady-state photosynthesis result in high levels of RuBP in wheat leaves (13). These levels exceed the number of binding sites for RuBP on the carboxylase by more than 2- to as high as 4- or more fold. In the dark, the levels of RuBP are about 10 to 20% of the number of binding sites. This has implications concerning the $K_{act}(CO_2)$ for activation of the carboxylase in the leaf. Based on the current literature (1, 8, 10, 12), the scheme given in Figure 1 indicates the mechanism of activation of the carboxylase as it might occur in the presence of effectors and RuBP.

The carboxylase enzyme (E) combines with CO_2 (C) by a slow reaction, followed by rapid binding with Mg^{2+} (M). The substrate RuBP (R) binds, followed by either the substrate CO_2 or O_2. Other positive or negative effectors (F) bind and alter the equilibrium of the equation by stabilizing the various complexes (1). A result of this stabilization is to change the concentration of Mg^{2+} and CO_2 necessary for half maximal activation of the enzyme ($K_{act}[CO_2]$) (5). Studies with the purified enzyme indicate that the apparent $K_{act}(CO_2)$ is dependent on pH, Mg^{2+}, and the presence of intermediates which serve as effectors and can bind competitively with RuBP at the active site. The heavy lines indicate the major route of change which the enzyme undergoes in the presence of CO_2, Mg^{2+}, and RuBP. Although included in the equation, the amounts of the E·C, E·C·R, and E·C·F forms are probably quite low compared to the other forms (12). Studies reported here suggest that in the light most of the enzyme is bound with RuBP either as E·R or E·C·M·R. At the other extreme, where the level of RuBP is lower than the number of binding sites (as in dark), other intermediates, which generally have lower binding affinities to the carboxylase than RuBP, will bind as the E·F and E·C·M·F forms. In the absence of these effectors, the apparent K_{act} (CO_2) is about 10 to 20 μM CO_2 (5). The K_{act} (CO_2) will decrease or increase depending on the ratio of the dissociation constants of binding of an effector to the activated E·C·M or inactive E forms (1). In the light with high RuBP, the K_{act} (CO_2) would be regulated by the presence of RuBP.

We have measured changes in activation of the RuBP carboxylase, the amounts of RuBP, and photosynthesis as indicated by CO_2 gas exchange to investigate how activation of the RuBP carboxylase is regulated *in vivo* in the light. Activation of the carboxylase in wheat leaves is dependent on irradiance but little affected by changes in CO_2 and O_2. Under very low CO_2 and O_2, activation of the RuBP carboxylase becomes comparatively unresponsive to changes in irradiance. Higher irradiance probably affects activation of the RuBP carboxylase by increasing pH and Mg^{2+} in the chloroplast stroma. The results indicate that, although pH and Mg^{2+} must be involved in moderating activation of the RuBP carboxylase in the light, changes in pH and Mg^{2+} cannot account entirely for the changes in activation. We propose that an effector, possibly RuBP, may be involved in modulating activation of the RuBP carboxylase in the light.

MATERIALS AND METHODS

Experiments were conducted using 7- to 8-d seedlings of durum wheat, cv 'Mexicali' grown in 6-ounce pots. CO_2 gas exchange was measured using a single pass IR gas analysis system as previously reported (13). The leaf temperature was monitored and controlled

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[3] Abbreviations: RuBP, ribulose 1,5-bisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CER, CO_2 gas exchange rate; PGA, 3-phosphoglycerate.

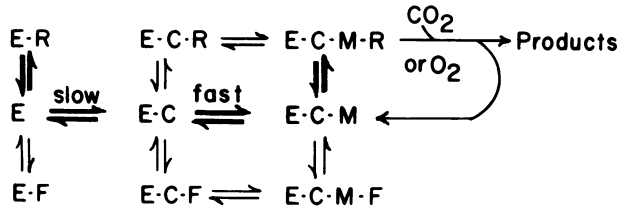

FIG. 1. Proposed mechanism of activation of the RuBP carboxylase as it might occur in the leaf. (E), RuBP carboxylase; (C), effector CO_2; (M), Mg^{2+}; (R), RuBP; (F), effector.

so that at all irradiances the leaf temperature was 26 ± 1°C. CO_2 concentrations were adjusted by mixing CO_2-free air or N_2 and 1 to 2% CO_2 in air using flowmeters. The mixtures were compared with a known standard. Internal CO_2 concentrations were calculated using the method of Wong *et al.* (20). The techniques for measurement and preservation of the *in vivo* activation of the RuBP carboxylase in leaf extracts were recently reported (14). The wheat leaves had about 3.0 mg Chl dm^{-2} or 40 cm^2 (g fresh weight)$^{-1}$.

Amounts of RuBP in the tissue were determined by adapting a previously reported technique (13). While the pot was still under a given irradiance and atmosphere, several wheat leaves were detached and rapidly frozen in liquid N_2. The procedures for RuBP measurement were optimized to reduce loss of RuBP (18). Within 20 min, the frozen leaves were ground to a powder in liquid N_2 and all Chl extracted in 80% (v/v) acetone at 0°C. Under these conditions, all RuBP remained with the insoluble leaf fragments. The almost-white leaf powder was suspended in 1 ml 5% $HClO_4$, 10 mg BSA was added, and the mixture extracted in a glass homogenizer at 0°C. The pellets were extracted twice more with 1 ml each of 5% $HClO_4$ and the extracts centrifuged. The RuBP from the leaves is best stored in the $HClO_4$ extract. After adding MOPS, to 50 mM, the extracts were stirred and 4 N KOH was slowly added to pH 6.5 to 7.0, and the $KClO_4$ removed. The RuBP was assayed for 30 min at 25°C in a reaction mixture of 313 μl containing 125 μl neutralized extract, 160 mM Bicine, 18 mM MOPS, 10 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (1 μCi[μmol]$^{-1}$), 0.1 to 0.15 mg tobacco RuBP carboxylase at pH 8.2. At least 85 to 95% of the RuBP originally in the leaf was measured by this technique, based upon the recovery of known amounts of added RuBP.

RESULTS

Steady state photosynthesis was measured using CO_2 gas exchange at various CO_2 and O_2 levels. The amount of RuBP was determined along with activation of the RuBP carboxylase (Figs. 2–4). We were unable to detect any effect of CO_2 between 100 and 1,400 μl(l)$^{-1}$ on activation of the RuBP carboxylase from intact wheat leaves (Figs. 2C and 3C). The amount of internal CO_2 as calculated by the stomatal resistance to CO_2 diffusion ranged from 3 to 50 μM at the external CO_2 levels used. This suggests that in the light of K_{act} (CO_2) value must have been lower than the dissolved CO_2 because CO_2 concentrations in this range had no effect on activation. This is in contrast to effects of CO_2 on the enzyme from dark-adapted wheat leaves as reported by Mächler (11).

The levels of RuBP in the leaves remained high and apparently saturating for CO_2 fixation at most levels of CO_2 and moderate to high irradiances (Fig. 3B). At extremes such as no CO_2 or dim light (100 μE[m^2·s]$^{-1}$), RuBP amounts in the leaf approached the number of binding sites on the RuBP carboxylase/oxygenase, which was about 100 nmol of binding sites (mg Chl)$^{-1}$ with these seedlings.

These observations are in direct contrast to the model of leaf photosynthesis as proposed by Farquhar (3, 4). In this model at

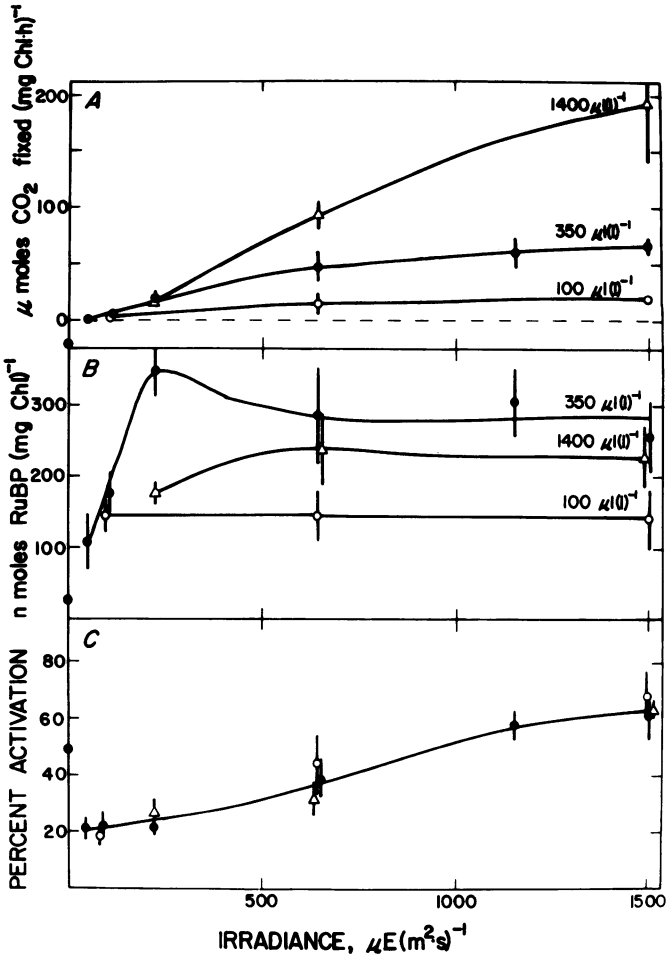

FIG. 2. Illumination of wheat seedlings in air and the effects on CER, RuBP levels, and activation of RuBP carboxylase with 100 μl(l)$^{-1}$ CO_2 (○), 350 μl(l)$^{-1}$ CO_2 (●), or 1,400 μl(l)$^{-1}$ CO_2 (△). Plants were brought to steady-state CER for at least 15 min before sampling. Each data point represents the average of at least five determinations. One SD about the mean is shown.

internal partial pressures less than 230 to 250 μl(l)$^{-1}$ CO_2, the rate of CO_2 assimilation is proposed to be saturated for RuBP, while at higher CO_2 partial pressures the rate is thought to be limited by RuBP regeneration. With our experiments at 650 μE(m^2·s)$^{-1}$ and 350 μl(l)$^{-1}$ external CO_2, the internal CO_2 calculated from stomatal diffusive resistances was about 280 μl(l)$^{-1}$ CO_2. The levels of RUBP did not appear limiting for photosynthesis up to 1,400 μl(l)$^{-1}$ CO_2.

Photosynthesis increased as expected when O_2 was reduced to less than 1.1% (Fig. 4A). Note that the levels of RuBP with 350 and 1,400 μl(l)$^{-1}$ CO_2 remained about the same, while RuBP greatly increased with 100 μl(l)$^{-1}$ CO_2 in low O_2 during steady-state photosynthesis (Fig. 4B). Indeed, under low O_2 and 100 μl(l)$^{-1}$ CO_2, RuBP was quite high under very dim light of 50 μE(m^2·s)$^{-1}$. Note with 100 μl(l)$^{-1}$ CO_2 and 0.1% O_2 that activation increased from 50 to 225 μE(m^2·s)$^{-1}$, but beyond that point did not respond to increased irradiance (Fig. 4C).

Changes in activation do require some minimal level of CO_2 and O_2. The addition of O_2 to 100 μl(l)$^{-1}$ CO_2 caused the percentage of activated enzyme to approach that shown by the higher CO_2 levels in Fig. 4C. At low irradiance (225 μE[m^2·s]$^{-1}$), addition of O_2 caused activation as well as CER to drop (Table I). There was a small decrease in the RuBP level. However, at high irradiance (1500 μE[m^2·s]$^{-1}$), addition of O_2 increased activation as well as CER with no change in RuBP levels. These increases in O_2

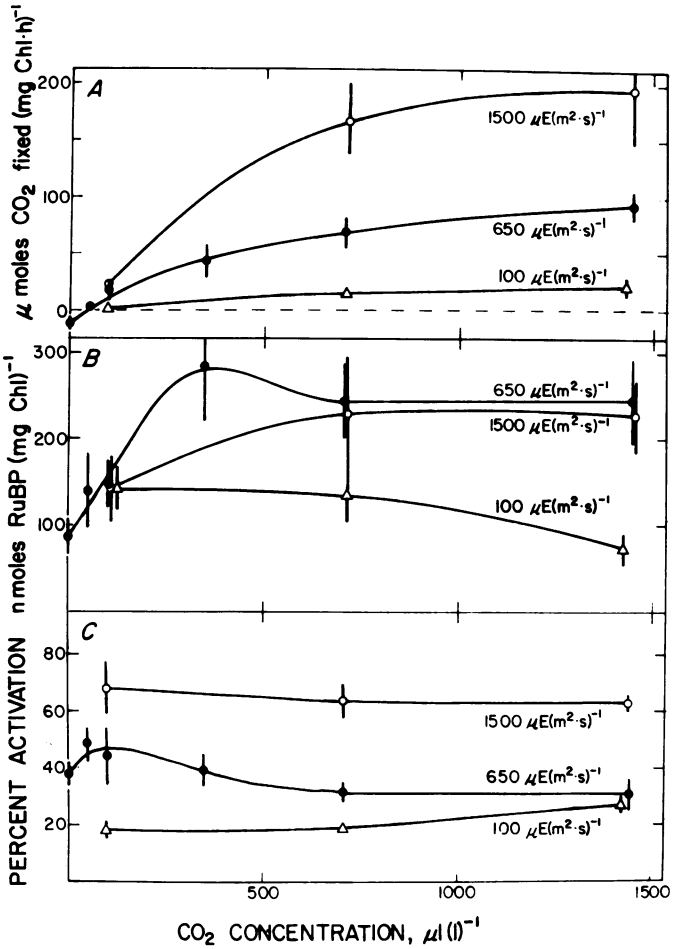

FIG. 3. Net rate of CO_2 uptake, RuBP levels, and activation of the RuBP carboxylase of wheat seedlings in air *versus* CO_2 concentration external to the leaf at various irradiances. Plants were brought to a steady-state for 15 min with white light at 1,500 $\mu E(m^2 \cdot s)^{-1}$ (○), 650 $\mu E(m^2 \cdot s)^{-1}$ (●), or 100 $\mu E(m^2 \cdot s)^{-1}$ (△) before sampling. Each point represents the average of five or more determinations. One SD about the mean is shown.

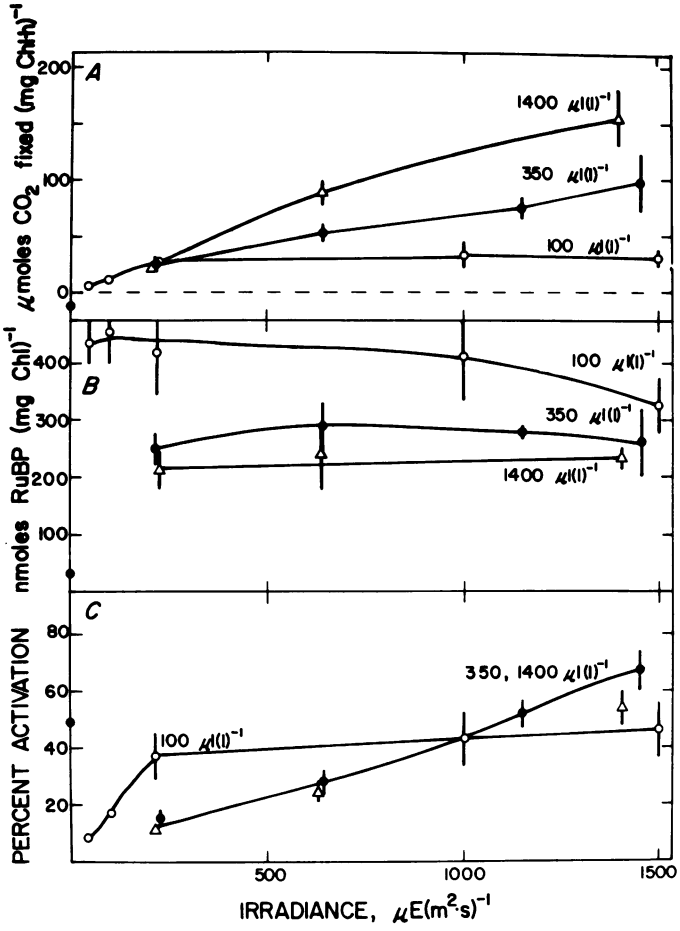

FIG. 4. Illumination of wheat seedlings in less than 1.1% O_2 and the effect on CER, RuBP levels, and activation of the RuBP carboxylase with 100 $\mu l(1)^{-1}$ CO_2, 0.1% O_2 (○), 350 $\mu l(1)^{-1}$ CO_2, 0.3% O_2 (●), or 1,400 $\mu l(1)^{-1}$ CO_2, 1.1% O_2 (△) in N_2. Each point represents the average of at least five determinations. One SD about the mean is shown.

concentration were too small to support photorespiration but could have helped poise the redox status of the electron transport chain as an acceptor of electrons. Note that photorespiration cannot be observed with 2 to 3% O_2 (21).

Comparison of the curves for activation (Figs. 2C and 3C) indicate that at low irradiance activation was substantially lower with low O_2. At high irradiance, activation was similar and not influenced by external O_2. Levels of RuBP were somewhat less with air at all irradiances with the most marked effect observed at 100 $\mu l(1)^{-1}$ CO_2. This was probably due to the combined effects of carboxylation and oxygenation consuming RuBP.

In an attempt to better understand the effects of CO_2 and O_2 in regulating activation of the RuBP carboxylase, wheat seedlings were brought to steady-state photosynthesis at 1,000 $\mu E(m^2 \cdot s)^{-1}$ in 350 $\mu l(1)^{-1}$ CO_2 and 0.3% O_2. The experiment was initiated by changing the gas phase to N_2 and removing CO_2 and O_2. The changes in RuBP and activation of the RuBP carboxylase were followed in light and dark. In the light with N_2 and the absence of external CO_2 and O_2, activation of the enzyme dropped to near zero while RuBP levels declined approaching 100 nmol(mg Chl)$^{-1}$ (Fig. 5). This level of RuBP was close to the calculated number of binding sites available on the carboxylase (13). However, if leaves were put in the dark with N_2, activation slowly decreased while RuBP levels dropped below 35 nmol(mg Chl)$^{-1}$.

Following treatment in the light with N_2 where activation was below 10% and RuBP was about 100 to 120 nmol(mg Chl)$^{-1}$), CO_2 and O_2 were added and activation increased again (Fig. 6). Note that even though O_2 is not thought to be an effector of the RuBP carboxylase, it did cause the enzyme to reactivate in the light. However, if the light was turned off when activation was low and RuBP was 100 to 120 nmol(mg Chl)$^{-1}$, subsequent addition of air levels of CO_2 or O_2 had no effect on activation.

The RuBP levels also increased when CO_2 or O_2 were given to the leaves in the light (Fig. 7). In the dark, addition of CO_2 or O_2 did not cause RuBP to decrease immediately but later at 30 min. The inability of the plants to utilize RuBP in the dark may have

Table I. *Influence of O_2 and Irradiance on Activation of the RuBP Carboxylase, CER at Steady-State, and Levels of RuBP at 100 $\mu l(l)^{-1}$ CO_2*

Irradiance	O_2	Activation of RuBP Carboxylase	CER	RuBP
$\mu E(m^2 \cdot s)^{-1}$		%	$\mu mol\ CO_2\ (mg\ Chl \cdot h)^{-1}$	$nmol\ (mg\ Chl)^{-1}$
225	0.05	37 ± 8	28.3 ± 2.4	418 ± 72
225	0.60	25 ± 4	15.9 ± 3.0	349 ± 23
1500	0.04	46 ± 10	29.2 ± 7.2	325 ± 50
1500	0.84	59 ± 7	41.9 ± 9.4	333 ± 71

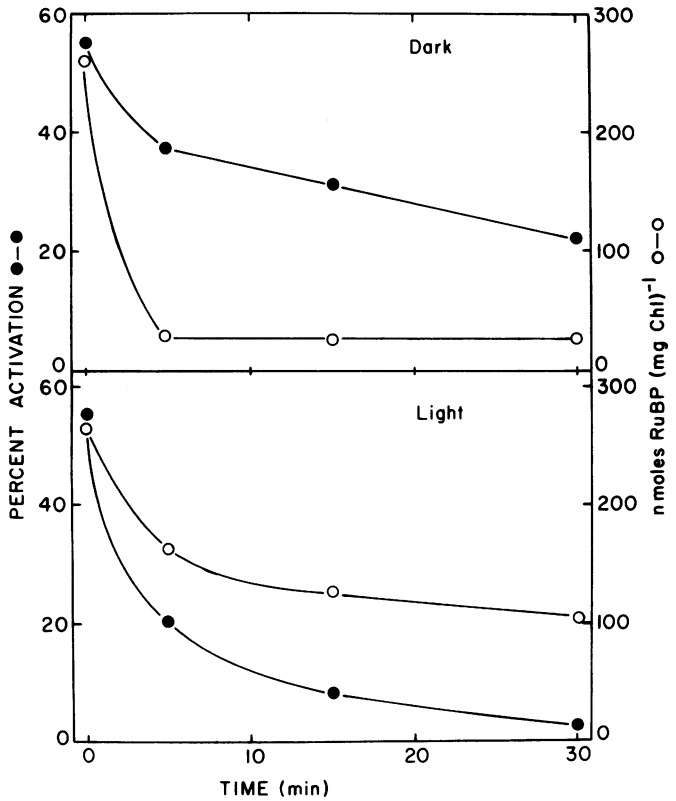

FIG. 5. Change in activation of the RuBP carboxylase (●) and levels of RuBP (○) of wheat seedlings in dark or light and N_2. Plants were brought to steady-state photosynthesis with 1,000 $\mu E(m^2 \cdot s)^{-1}$ white light and 350 $\mu l(l)^{-1}$ CO_2, 0.3% O_2 before the experiment began. At time zero, the gas phase was changed to N_2 and the lights were either turned off or left at 1,000 $\mu E(m^2 \cdot s)^{-1}$. By 30 min, the internal CO_2 had dropped to less than 5 $\mu l(l)^{-1}$.

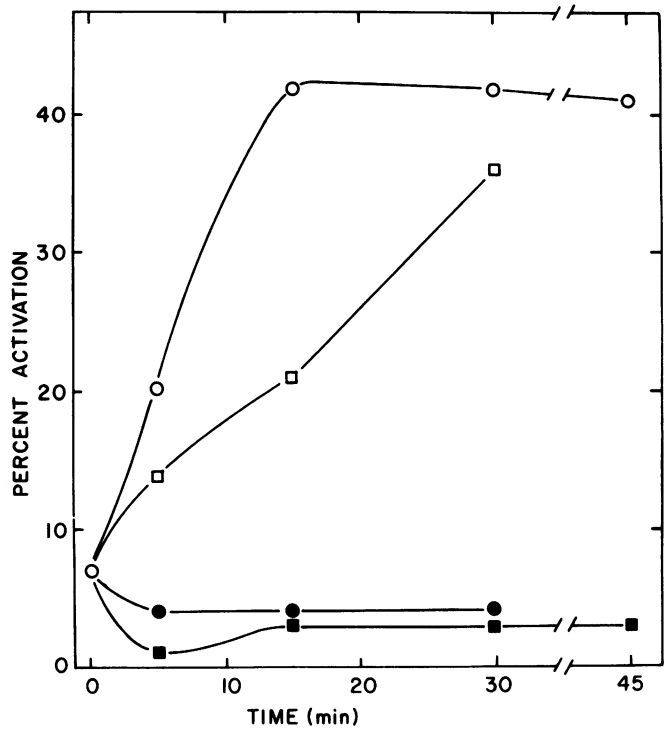

FIG. 6. CO_2 and O_2 in light or dark on activation of the RuBP carboxylase in wheat seedlings. Plants were brought to steady-state photosynthesis with 1,000 $\mu E(m^2 \cdot s)^{-1}$ white light, 350 $\mu l(l)^{-1}$ CO_2, and 0.3% O_2. The gas was then switched to N_2 and the plants further illuminated for another 15 min (see Fig. 5, light) before beginning the experiment. At time zero, the gas phase was changed to either 350 $\mu l(l)^{-1}$ CO_2, 0.3% O_2 (○) or 21% O_2 (□) in light or 350 $\mu l(l)^{-1}$ CO_2, 0.3% O_2 (●) or 21% O_2 (■) in dark, and activation of the RuBP carboxylase was measured.

been caused by the almost complete deactivation of the RuBP carboxylase.

DISCUSSION

Effect of CO_2 on Activation. In 21% O_2, the degree of activation of the RuBP carboxylase appeared independent of CO_2 concentration, while activation was altered by irradiance. In the dark, a positive correlation of CO_2 concentration with enzyme activation has been reported with intact wheat leaves (11) and spinach chloroplasts (2). The obvious difference between light and dark is the presence of high amounts of RuBP in the light. This could reflect *in vivo* the observation with the purified enzyme where $K_{act}(CO_2)$ is greatly reduced in the presence of effectors, including RuBP (5). As the observations suggest, the CO_2 levels used in these experiments were not limiting for activation under the irradiance utilized. The $K_{act}(CO_2)$ of the RuBP carboxylase in the leaf apparently exhibits two values; one which exists in the dark where RuBP is low (11), and another having a considerably lower $K_{act}(CO_2)$ occurring in the light where RuBP levels are high.

Role of pH and Mg^{2+}. Studies with the purified enzyme indicate that activation of the RuBP carboxylase depends on pH and Mg^{2+} levels (5, 10, 19). However the involvement of only these two parameters as major factors of control of activation of the RuBP carboxylase in leaves in the light does not always appear to fit the observations. One point of disagreement is seen upon comparison of activation in low light and dark. The pH and Mg^{2+} gradients are thought to be lowest in the dark (7, 15, 16, 21); hence, activation ought to be the lowest in the dark. This is not the case, because activation is lowest at low light intensities, while after 10

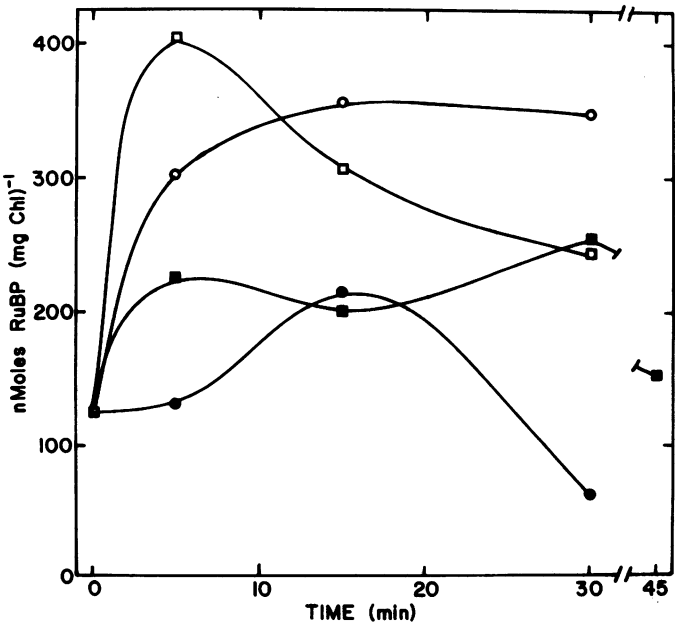

FIG. 7. CO_2 and O_2 in light or dark on levels of RuBP after they had been brought to the binding site number of the RuBP carboxylase in wheat seedlings. As described in Figure 6, plants were brought to the pretreatment conditions of 15 min of Figure 5 in light before beginning the experiments and measuring the levels of RuBP. Symbols as given in Figure 6.

h dark, activation is higher (about 50%) (Ref. 13; Fig. 2). Something in addition to pH and Mg^{2+} gradients must be involved in regulation of activation of the RuBP carboxylase. A negative effector of the carboxylase could explain these results. Under suboptimal Mg^{2+} and CO_2 concentrations, the activation of the RuBP carboxylase is influenced by the presence of effectors (1, 5, 12). These effectors appear to bind only at the same binding site as RuBP (1, 12). The most likely negative effector found in sufficient amounts is RuBP itself. In light, RuBP is found in greater concentration than the binding sites of the carboxylase. In low light, Mg^{2+} may be low and the stroma pH not as favorable so that the inactive E·R form of the enzyme is preferred. In high light where Mg^{2+} and pH are more optimal, activation of the carboxylase favors more E·C·M·R.

Krause has shown that changes in light scattering and Chl *a* fluorescence of chloroplasts, either isolated or in the leaf, are related to light-induced intrachloroplast Mg^{2+} fluxes (6, 7). Efflux of Mg^{2+} from the thylakoids to the stroma occurs secondary to active H^+ uptake by the thylakoids. When the pH and Mg^{2+} gradients in the chloroplast are large, the light scattering is maximal and the fluorescence is minimal at steady-state. As the magnitude of the proton gradient is related to the adenylate phosphorylation state, chloroplast light scattering and fluorescence also serve as indicators of the stroma phosphorylation potential, [ATP]/[ADP] [Pi]. When spinach leaves were illuminated either in N_2 or air, the light-induced intrachloroplast Mg^{2+} and pH gradient as indicated by chloroplast light scattering and fluorescence were suppressed with increasing CO_2 levels (6). We observed that increasing CO_2 at constant irradiance did not change activation of the RuBP carboxylase (Fig. 4C). Activation does not always follow the expected changes in pH and Mg^{2+} across the thylakoid membranes.

We do not wish to conclude that changes in pH and Mg^{2+} in the chloroplast stroma have little relationship to activation of the RuBP carboxylase. Indeed, the rise in activation which follows a rise in CO_2 gas exchange as irradiance is increased (Figs. 2 and 4) could suggest that increased irradiance is causing more favorable pH and Mg^{2+} levels resulting in increased activation. Although activation of the RuBP carboxylase can be controlled by changes in Mg^{2+} and pH, these factors alone are not sufficient to explain completely its regulation in the leaf.

Need for CO_2 or O_2 for Activation. Methods which alter activation, such as illumination in N_2 atmosphere, can serve as probes for the mechanism of regulation of activation of the carboxylase. Note that with no added CO_2 and O_2 in light (Fig. 5), activation approaches zero while RuBP approaches the number of binding sites available on the enzyme. This indicates that E·R is being formed at the expense of E·C·M·R thereby sequestering the RuBP (19) from attack by active enzyme. The rate of enzyme deactivation was much slower in the dark than in the light suggesting a different mechanism is involved in each case. In darkness, Mg^{2+} concentration in the chloroplast stroma should be lower than in light (15). The RuBP was consumed by the remaining active enzyme, as the effector CO_2 dissociated from the active enzyme complex with slow formation of the inactive E form. In light, RuBP dropped only to the number of binding sites as E·R was formed. It is doubtful that the dissolved CO_2 was significantly different between the light- and dark-treated leaves as the stomatal resistance and the rate of CO_2 evolution were similar (data not shown).

Although CO_2 serves as an effector of the enzyme, there is no evidence that O_2 is similarly involved (9). Addition of O_2 in the absence of CO_2 in the light did support activation which was not observed in the dark (Fig. 6). Apparently, the presence of an electron acceptor (*i.e.* CO_2 and O_2) is required in the light. In the absence of external electron acceptors, the regeneration of ATP and NADP must decrease as the photosystems become reduced. Oxygen in the chloroplast mediates pseudocyclic electron flow with production of ATP, which then could maintain RuBP levels using the cell reserves for carbon. Regeneration of NADPH would depend both on PGA production and action of the photorespiratory pathway (7). The slower rate of activation observed after introduction of O_2 compared with that of CO_2 probably reflects the low CO_2 concentration present in the leaf in the former case. Calculated CO_2 concentrations within the leaf were 25, 31, and 22 $\mu l(1)^{-1}$ at 5, 15, and 30 min, respectively (Fig. 6), and may have been too low to allow a more rapid activation of the enzyme.

A large increase in RuBP level seen following introduction of O_2 or CO_2 (Fig. 7) probably represents the resumption of electron flow allowing the Calvin cycle to turn. In the presence of CO_2, the RuBP level stabilized at about 340 nmol(mg Chl)$^{-1}$, whereas with O_2 the level dropped, probably resulting from the drain of carbon from the cycle brought on by oxygenation.

After deactivation of the leaf carboxylase by illumination in N_2, addition of CO_2 in wheat did restore activation (Fig. 6), although it did not return to the level of the control (Fig. 5). Photoinhibition, due to deprivation of CO_2 and O_2 at high irradiance, may explain why activation did not recovery fully (17). If recovery of activation was limited only by availability of CO_2 for recombination with the carboxylase, a similar increase in activation should have been seen in dark as well as light (Fig. 6). That this was not the case indicates again that photosynthetic electron flow does influence activation of the carboxylase. Even though addition of CO_2 or O_2 in dark did not change activation, they did provide for an increase in RuBP in the dark (Fig. 7).

Changes in activation of the RuBP carboxylases in the leaf appears to require something more than pH, Mg^{2+}, or CO_2 changes. Such a candidate may be RuBP acting as a negative effector. Because activation changes also occur in low O_2, at levels unable to support photorespiration, the effector is probably not connected with the photorespiratory pathway. In dark, where RuBP is low, changes in CO_2 levels cause apparent changes in activation. However, in the light where RuBP is high, the levels of Mg^{2+} and possibly pH most likely account for changes in activation of the RuBP carboxylase by changing the ratios of the E·R and E·C·M·R forms of the enzyme. Future research combining measurements of RuBP and other tissue metabolites with effector studies on the purified enzyme should determine these possibilities.

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